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The mechanism of inhibition of cancer cell proliferation by vitamin A is poorly understood because many of the targets that mediate the retinoid-dependent growth suppression are not known. We have recently identified a novel retinoid-responsive gene target, **TIG3**, that we believe may be a key player in mediating the retinoid-dependent suppression of tumor cell proliferation. Understanding the mechanism of TIG3 action may provide insights that lead to innovative new anti-breast cancer therapies. Exploiting this potential requires that we understand how TIG3 inhibits cell proliferation – the major goal of this proposal. ***Specific Aim 1*** Subcellular location plays a major role in determining function, and knowing location provides clues about function. Therefore, our first goal is to localize TIG3 in breast cancer cells. ***Specific Aim 2*** Most proteins contain distinct functional domains, some responsible for localization and others for function. We hypothesize that TIG3 is divided into distinct functional domains that are important for growth suppression. We are testing this hypothesis. ***Specific Aim 3*** The TIG3 amino acid sequence/structure reveals no obvious catalytic functional domains, suggesting that TIG3 acts by modulating the function of other proteins. A major goal of the study is to identify these targets. During the first year we have 1) constructed a plasmid-based TIG3 expression systems and used it to express TIG3 in cells, 2) identified a perinuclear localization of TIG3 in cells, 3) demonstrated that the TIG3 carboxy-terminal hydrophobic domain guides appropriate subcellular localization, 4) shown that the TIG3 carboxy-terminal tail is required for optimal cell killing, and 5) constructed an adenovirus expression system that permits efficient TIG3 expression for biochemical studies.

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INTRODUCTION

Breast cancer is a serious disease that affects millions of women. Vitamin A and related retinoids are important dietary components that are important for normal epithelial cell proliferation and differentiation and are thought to be chemopreventive against development of cancer. Analogs of vitamin A have been shown to be efficacious for treatment in the mammary gland and breast cancer systems, and have been shown to inhibit the proliferation of cultured breast cancer cells. However, the mechanism of inhibition of cancer cell proliferation by retinoids is poorly understood because many of the targets that mediate the retinoid-dependent growth suppression are not known. We have recently identified a novel retinoid-responsive gene target, **TIG3**, that we believe may be a key player in mediating the retinoid-dependent suppression of tumor cell proliferation. Treatment of $T_{47}D$ human breast cancer cells with retinoid increases TIG3 mRNA levels, an increase that is associated with a suppression of cell proliferation. TIG3 mRNA level, in contrast, is not increased nor is growth suppressed in a retinoid-insensitive line derived from MCF-7 cells. These results suggest that TIG3 may mediate the retinoid-dependent suppression. For these reasons we hypothesis that TIG3 may be a key mediator of retinoid-dependent tumor suppression in breast cancer. Understanding the mechanism of TIG3 action may provide insights that lead to innovative new anti-breast cancer therapies. Exploiting this potential requires that we understand how TIG3 inhibits cell proliferation – the major goal of this proposal. ***Specific Aim 1*** Subcellular location plays a major role in determining function, and knowing location provides clues about function. Therefore, our first goal is to use immunological and cell fractionation methods to localize TIG3 in breast cancer cells. ***Specific Aim 2*** Most proteins contain distinct functional domains, some responsible for localization and others for function. We hypothesize that TIG3 is divided into distinct functional domains that are important for growth suppression. To test this, we will construct a series of mutants and measure the ability of each mutant to suppress growth using a breast cancer cell colony formation assay. ***Specific Aim 3*** The TIG3 amino acid sequence/structure reveals no obvious catalytic functional domains, suggesting that TIG3 acts by modulating the function of other proteins. The goal of this specific aim is to identify candidate target proteins using co-immunoprecipitation, affinity chromatography and two-hybrid screening. The candidate target proteins will be identified by peptide microsequencing (affinity chromatography) or gene cloning and sequencing (two-hybrid cloning). It is expected that these studies will reveal important clues about the TIG3 mechanism of action. It is our hope that understanding this how TIG3 signals growth suppression will lead to innovative new anti-breast cancer therapies.

BODY

Specific Aims 1 & 2

- *Subcellular location plays a major role in determining function, and knowing location provides clues about function. Therefore, our first goal is to use immunological and cell fractionation methods to localize TIG3 in cells.*
- *Most proteins contain distinct functional domains, some responsible for localization and others for function. We hypothesize that TIG3 is divided into distinct functional domains that are important for growth suppression. To test this, we will construct a series of mutants and measure the ability of each mutant to suppress growth using a breast cancer cell colony formation assay.*

The studies outlined in the first two specific aims are most efficiently described when combined. Thus, we will describe the construction of our initial set of mutants, their detection in cells, and their

subcellular distribution in this section. One manuscript has been published based on this work, and several more are planned. The work is summarized below.

Construction of TIG3 inducible adenovirus expression constructs TIG3 is an 18 kDa 164 amino acid growth suppressor protein that is present in very low levels in cells. An important goal of this study is to identify the role of various functional domains within the protein and to determine which domain controls subcellular localization. The sequence of TIG3 is shown in **Fig. 1**.

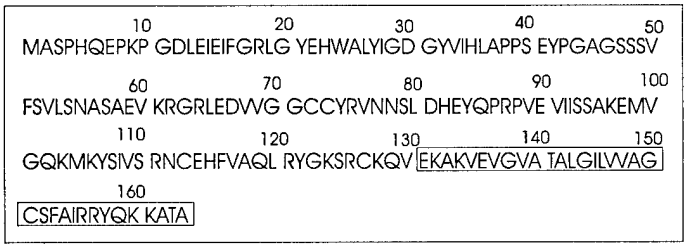


Fig. 1 *Structure of TIG3. The amino terminal segment encodes amino acids 1-134, while the carboxy terminal tail includes amino acids 135-164.*

The protein is divided into an amino-terminal domain and a carboxy-terminal hydrophobic domain. We hypothesize that TIG3 subcellular distribution is controlled by the carboxy-terminal hydrophobic domain and that this serves to anchor TIG3 to membranes. We further hypothesize that various conserved domains in the amino terminus are required to mediate the growth inhibitory effects. To test this idea we constructed the mutants shown in **Fig. 2**. $TIG3_{1-164}$ encodes the full-length protein, while $TIG3_{1-134}$ encodes the TIG3 amino-terminus but lacks the carboxy-terminal tail. In the previous report, we described studies showing that $TIG3_{1-164}$ efficiently inhibits proliferation, while $TIG3_{1-134}$ is minimally effective {2613}. Thus, these studies showed that the carboxyl terminal domain is required for activity. These studies also revealed that TIG3 is localized in the perinuclear region in cells {2613}. These early studies were performed using plasmid-based expression systems that were not very efficient in that transfection would only target 10% of cells in a dish. This plasmid-based system required elaborate selection protocols to quantitate cell kill results {2613}. To circumvent this problem, we subcloned full-length TIG3 ($TIG3_{1-164}$), and all of the other mutants shown in **Fig. 2** into an inducible adenovirus vector, tAd5. This expression system relies on infection with two viruses – one virus that expresses the protein of interest, and a second virus that produces the TET activator protein (Ad5-TA). The TET activator (TA) binds to the promoter in the expression virus to regulate expression. In addition, the TET activator can be inactivated when tetracycline is present in the medium. Thus, expression of TIG3 can controlled in a regulated manner. This system has two major advantages. First, it delivers protein by infection and, therefore, virtually 100% of the cells can express the protein.

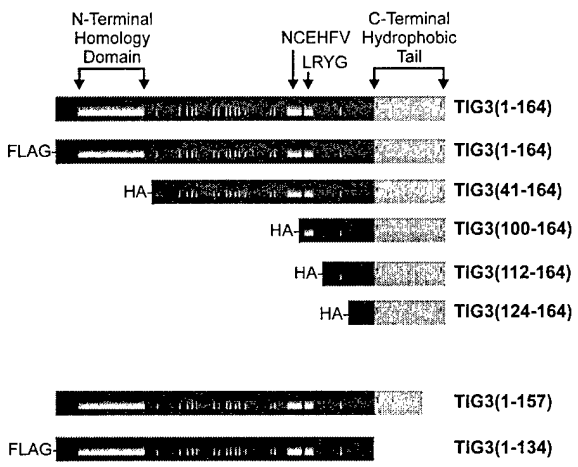
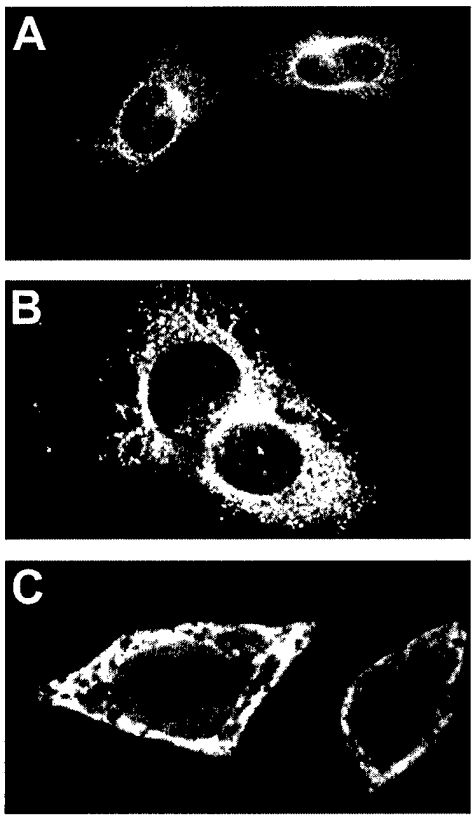


Fig. 2 *TIG3 expression constructs. The various TIG3 encoding segments were synthesized using PCR and then cloned into the tAd5 adenovirus. The resulting recombinant adenoviruses encode the indicated TIG3 mutants. The top construct is full-length TIG3 ($TIG3_{1-164}$). Each of the other constructs has been modified by truncation from the carboxyl or amino-terminal ends. In addition, we have added specific epitope tags to the amino terminal end in most of the constructs (FLAG or HA). Antibodies are available that detect each of these epitopes. The vertical hatches represent areas of conservation with other members of the TIG3 family of proteins {2613}.*

Second, the level of TIG3 per cell can be regulated by the level of tetracycline present in the cell culture medium. Additional studies (not shown) indicate that each of the mutant TIG3 proteins encoded by the viruses shown in **Fig. 2** are expressed in virus-infected breast cancer cells. Expression was detected by immunoblot using anti-FLAG, anti-HA, and/or anti-TIG3 antibodies. In addition, each virus was optimized regarding infection ratio etc. so that 100% of the cells are infected and express the TIG3 protein.



TIG3 subcellular localization- immunofluorescent detection In the previous report, we demonstrated that full-length TIG3 localizes in the perinuclear region in cells. **Fig. 3** shows the results for CHO cells. A similar subcellular distribution of $TIG3_{1\text{-}164}$ and $TIG3_{1\text{-}134}$ was observed using the adenovirus-based delivery in a variety of cells, including $T_{47}D$ breast cancer cells and SK-BR-3 cells.

Fig. 3 *Subcellular localization of $TIG3_{1\text{-}164}$ and $TIG3_{1\text{-}134}$ in CHO cells. Cells were transfected with expression plasmid encoding $TIG3_{1\text{-}164}$ (panels A and B) or $TIG3_{1\text{-}134}$ (panel B). Cells in panel B were additionally treated with cycloheximide {2613}. CHO cells were chosen as an initial test cell since they are easily transfected.*

Regulation of breast cancer cell proliferation by TIG3 We have recently evaluated the effects of adenovirus-mediated delivery of $TIG3_{1\text{-}164}$ on proliferation of a variety of breast cancer cell lines. For these studies, cells were plated in 35 mm dishes and allowed to attach overnight. The cells were then infected at a multiplicity of infection (MOI) of 0 to 150 with tAd5-$TIG3_{1\text{-}164}$ for 12 h. The virus was then removed and incubation was continued for an additional 60 h at which time the cells were harvested and counted. **Fig. 4A** shows that SK-BR-3 cell number is dramatically reduced by $TIG3_{1\text{-}164}$ expression. Cell number is 50% reduced at an MOI = 50, and 90% at MOI $\geq$ 75. **Fig. 4B** shows that the growth suppression is directly correlated with increased $TIG3_{1\text{-}164}$ expression.

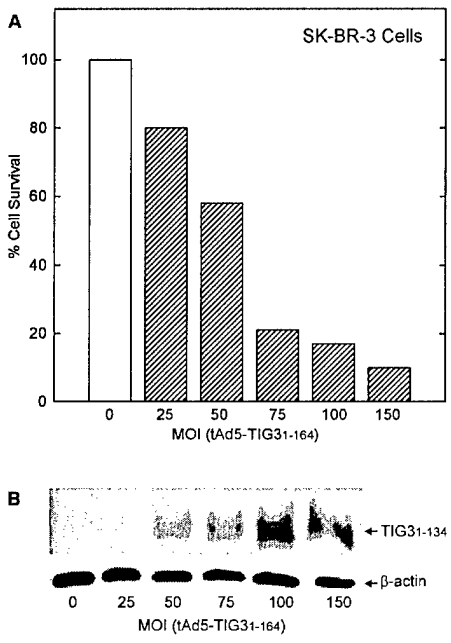


Fig. 4 *TIG3 reduces SK-BR-3 cell survival. **A** SK-BR-3 cells were plated, permitted to attach overnight, and then treated with 0 – 150 MOI of tAd5-$TIG3_{1\text{-}164}$ for 12 h and the virus was removed. After an additional 60 h, the cells were harvested and counted. Percent cell survival at each MOI is calculated by comparison to a group containing an identical amount of tAd5-EV (empty virus). **B** Total cell extracts were prepared from cells treated as above, and then electrophoresed on an 8% gel for immunoblotting with rabbit anti-human TIG3. β-actin was included as a control blot to assure equal loading of protein.*

Additional studies show that $TIG3_{1-134}$, a mutant that lacks the carboxy-terminal tail, is not an effective growth suppressor (not shown). This suggests that the carboxyl-terminal tail is essential for function. We are presently in the process of testing the other TIG3 mutants indicated in **Fig. 2** in the growth suppression/cell death assay. At the end of this series of experiments, we expect to have identified important functional domains within the TIG3 protein that mediate growth inhibition.

To determine whether the response is reversible, we treated SK-BR-3 cells with 75 MOI of $TIG3_{1-164}$-encoding virus for 12 h. This level of $TIG3_{1-164}$ produces optimal suppression of proliferation (see **Fig. 4A**). The virus was then removed, and cell growth was then continued for the times indicated in **Fig. 5**. It is clear from this experiment that the cells do not rapidly recover from the treatment with $TIG3_{1-164}$, as growth remains markedly suppressed at day four.

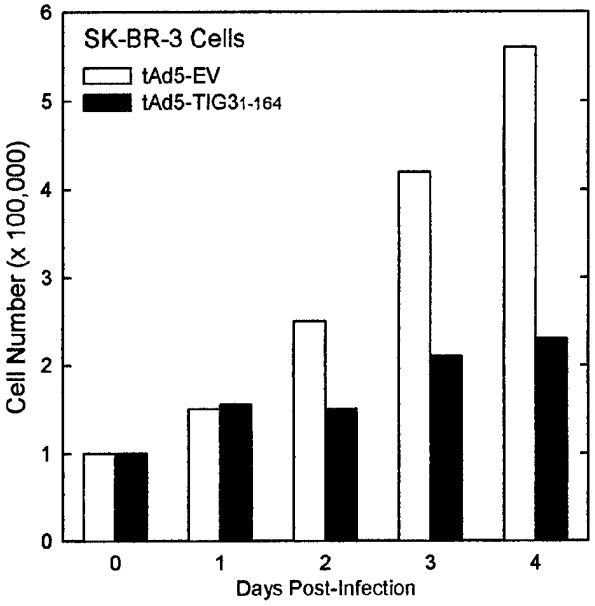


Fig. 5 *SK-BR-3 cells were treated for 12 h with 75 MOI of tAd5-$TIG3_{1-164}$ or tAd5-EV (empty vector). The virus was then removed, and incubation was continued for theindicated number of days post-infection. At each time point the cells were harvested and cell number was determined.*

<u>TIG3 promotes apoptosis</u> A major goal of this study is to begin to understand the mechanism whereby TIG3 suppresses human breast cancer cell proliferation. The results summarized in **Fig. 4A** suggests that the total number of cells in the culture dish is diminished by $TIG3_{1-164}$ treatment. This suggests that that in addition to inhibiting cell growth, TIG3 actually reduces the number of cells below the starting level. This suggests that TIG3 actively causes cell death.

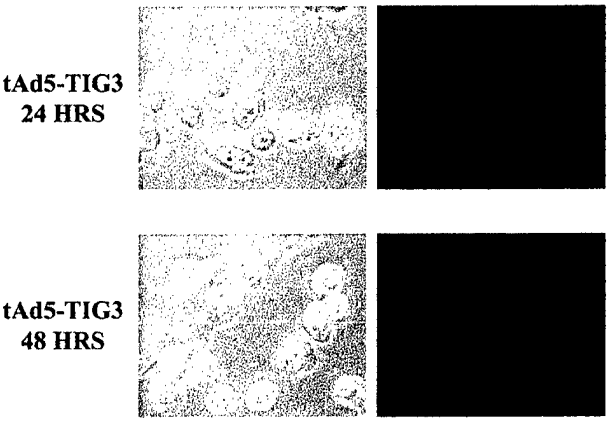


Fig. 6 *TIG3 expression causes nuclear condensation. SK-BR-3 cells were infected with adenovirus encoding $TIG3_{1-164}$ for 12 h. Fresh medium was added and at 24 h and 48 h cells were harvested, fixed (2% paraformaldehyde for 20 min at RT, 100% methanol for 10 min at 4 C), and stained with 1 μg/ml Hoechst 33258 stain for 10 min at room temperature. Corresponding bright field (left panels) and fluorescent photographs (right panels) are compared. All cells expressed $TIG3_{1-164}$ as detected by immunoblot (not shown). Note the compression of many of the nuclei in the TIG3-expressing cells at 48 h. No nuclear compaction was observed in cells expressing the control (empty) adenovirus (not shown).*

For this reason, we have examined the hypothesis that TIG3 promotes apoptosis. **Fig. 6** shows an experiment in which SK-BR-3 cells were treated for 24 or 48 h with tAd5-TIG3$_{1-164}$. All cells in the tAd5-TIG3$_{1-164}$ group expressed TIG3 as measured by immunohistology (not shown). The left panel in each set shows the bright field image of the cell field. The right panel shows the Hoechst-stained nuclei. It is important to note that the nuclei of the cells in the tAd5-EV group are of normal size (not shown). In contrast, the nuclei in many of the cells in the tAd5-TIG3$_{1-164}$-infected group at 48 h are reduced in size. These images show that the nuclei are compacted in TIG3-expressing cells, a mark of undergoing apoptosis. In addition, preliminary studies suggest that PARP cleavage occurs in TIG3-expressing cells, providing further evidence that TIG3 induces apoptosis.

Specific Aim 3

- *The TIG3 amino acid sequence/structure reveals no obvious catalytic functional domains, suggesting that TIG3 acts by modulating the function of other proteins. The goal of this specific aim is to identify candidate target proteins using co-immunoprecipitation, affinity chromatography and two-hybrid screening.*

Development of an adenovirus-based TIG3 expression system We have proposed that TIG3 binds to target proteins to regulate cell proliferation and survival. A major goal of this study is to identify proteins that interact with TIG3, in order to understand its mechanism of action. We initiated these studies by expressing TIG3 in cells and preparing cell extracts that could be used for TIG3 antibody pull down experiments. However, a major problem with these studies was the inability to express enough TIG3 protein to make these biochemical experiments possible. This was due to the use of a plasmid-based expression system, and the fact that TIG3 kills cells very efficiently and limits the number of TIG3-expressing cells that can be obtained. To circumvent these difficulties we switched from the plasmid-based to an adenovirus-based expression system. The use of this system has solved the problem of minimal protein expression. In addition, it has solved the problem of cell viability, since we are using an inducible expression system that permits the level of TIG3 to be controlled. We are presently using the adenovirus-based expression system to identify proteins that interact with TIG3. In these experiments, we express TIG3 at high levels in SK-BR-3 breast cancer cells, treat the cells with crosslinking agent (DSP) and then immunoprecipitate the crosslinked complexes. We have affixed HA- and FLAG- epitope tags to the amino terminus of the expressed TIG3 proteins as precipitation anchors (see **Fig. 2**).

KEY RESEARCH ACCOMPLISHMENTS

Previous Report Period

- We have constructed plasmid-based TIG3 expression systems and used these to express TIG3 in cells (*Specific Aims 1 and 2*)

- We have constructed our first TIG3 mutant, TIG3$_{1-134}$ and begun to study its function (*Specific Aims 1 and 2*)

- We have shown that TIG3 assumes a perinuclear location in cells (*Specific Aim 1*)

- We have demonstrated that the TIG3 carboxy-terminal hydrophobic domain is required for appropriate subcellular localization (*Specifc Aim 1*)

- Eliminating the TIG3 carboxy-terminal tail reduces the ability of TIG3 to kill cells (*Specific Aim 2*)

- TIG3 kills breast cancer cell lines (e.g., $T_{47}D$) (*Specific Aim 2*)

Present Report Period

- We have extended our results to show that TIG3 expression kills SK-BR-3 breast cancer cells and our findings suggest that TIG3 promotes breast cancer cell apoptosis (*Specific Aim 1*)

- Adenovirus expression systems have been constructed that permit more efficient studies of cell killing and permits efficient production of TIG3 in cells for biochemical studies (*Specific Aims 1, 2 and 3*). This virus produces high-level expression of TIG3 in MCF7 cells.

- We are presently working to studying the ability of a series of TIG3 mutants to suppress SK-BR-3 growth with the goal of identifying the functional domains that are important for cell killing.

- TIG3 appears to promote apoptosis in SK-BR-3 breast cancer cells.

REPORTABLE OUTCOMES

- We have a manuscript published, and two additional manuscripts will be submitted layer this year.

- Anne Deucher and Mike Sturniolo will be reporting on these TIG3 studies as part of their Ph.D. theses

- Shervin Dashti, who completed a number of the early TIG3 studies, has completed his Ph.D.

- We have developed adenoviral TIG3-producing vectors that may be useful for gene therapy

CONCLUSIONS

We consider this work to be very important from the point-of-view of future breast cancer therapeutics. Our studies completed to date clearly show that TIG3 inhibits the breast cancer cell proliferation. We also suspect that TIG3 has the ability to kill (cause apoptosis) breast cancer cells independent of its effects on cell proliferation. We expect that by the time these studies are concluded we will have isolated the active domains within the protein that are responsible for these events, and that we will have also tested the ability of TIG3 to kill cells *in vivo* in a human tumor cell model system.

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Deucher A, Nagpal S, Chandraratna AS, DiSepio D, Robinson NA, Dashti SR, Eckert RL (2000) The carboxyl-terminal hydrophobic domain of TIG3, a class II tumor suppressor protein, is required for appropriate cellular localization and optimal biological activity. Inter. J. Oncology 17:1195-1203.